Parasitol Res (2004) 93: 30–35
DOI 10.1007/s00436-004-1088-6

ORIGINAL PAPER

**S. A. Oliveira · L. M. Silva · A. A. Barbosa Júnior
R. Ribeiro-dos-Santos · E. M. Coutinho · Z. A. Andrade
M. B. P. Soares**

Decreased humoral and pathologic responses in undernourished mice infected with *Schistosoma mansoni*

Received: 18 November 2003 / Accepted: 3 February 2004 / Published online: 30 March 2004
© Springer-Verlag 2004

Abstract We previously demonstrated that mice subjected to a hypoproteinic diet showed milder chronic lesions on infection with *Schistosoma mansoni* than normally fed mice. Here we compare the immune response of well-nourished and undernourished mice with chronic *S. mansoni* infection. The proliferative response and cytokine (IFN-γ and IL-5) production of splenocytes from undernourished mice against the soluble egg antigen (SEA) of *S. mansoni* or concanavalin A was similar to that of well-nourished mice. The levels of SEA-specific IgG1, IgG2b and IgG3 antibodies were significantly higher in the sera of well-nourished mice in comparison with undernourished mice. Undernourished animals also exhibited diminished periovular granuloma size compared to well-nourished infected controls. Our results support the importance of host nutritional status in the humoral immune response of mice and its effects on the development of periovular granulomas in malnourished animals infected with *S. mansoni*.

Introduction

Schistosomiasis mansoni is an important world health problem, especially in developing countries, where it is usually associated with an impaired nutritional status. The severe form of the disease (hepatosplenic form) results from the immuno-inflammatory response against schistosome eggs deposited in the intrahepatic periportal veins, causing the development of periportal fibrosis and liver malfunction (Warren 1966). Although *Schistosoma mansoni* infection aggravates the nutritional status in human beings, the pathological manifestations of schistosomiasis in undernourished individuals appear less severe than in well-nourished patients (Coutinho et al. 1972, 1997a).

An experimental model to induce malnutrition in mice infected with *S. mansoni* has been developed in our laboratory by feeding Swiss Webster mice with a hypoproteinic diet, referred to as the "regional basic diet" (RBD) which is related to the food habits of poor income people residing in an endemic area of north-eastern Brazil (Coutinho 1980; Coutinho et al. 1997b). In this situation, undernourished mice develop smaller periovular granulomas but not the periportal fibrosis observed in about 40% of well-nourished infected controls (Andrade and Cheever 1993; Coutinho et al. 1997b).

Fibrosis development is the result of a balance between collagen deposition and its degradation by catalytic enzymes (Burt 1993). These phenomena are regulated by immune responses through the production of soluble mediators which affect different cell types, stimulating or inhibiting both the production of collagen and of collagenases. In *S. mansoni* infection, modulation of granuloma formation and collagen deposition are associated with a Th2 response stimulated by the production of schistosome egg antigens (Andrade and Warren 1964; Wynn and Cheever 1995). The treatment of *S. mansoni*-infected mice with interferon (IFN-γ) causes a decrease in granuloma size and fibrosis deposition (Czaja et al. 1989). Since immune responses are usually depressed in undernourished individuals (Machado 1983; Chandra 1997), this paper deals with the importance of the host's nutritional status on the immune response to schistosome antigens in undernourished mice chronically infected with *S. mansoni*, and its influence on the development of periovular granulomas and hepatic fibrosis in these animals.

S. A. Oliveira · E. M. Coutinho
Centro de Pesquisas Aggeu Magalhães,
Fundação Oswaldo Cruz, Recife, PE, Brazil

L. M. Silva · A. A. Barbosa Júnior · R. Ribeiro-dos-Santos
Z. A. Andrade · M. B. P. Soares (✉)
Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz,
Rua Waldemar Falcão 121, Brotas,
40295-001 Salvador, BA, Brazil
E-mail: Milena@cpqgm.fiocruz.br
Tel.: 55-71-3564320
Fax: +55-71-3564320

Materials and methods

Animals

Three-week-old Swiss Webster mice (male and female) were raised and maintained at the animal facilities of the Aggeu Magalhães Research Center Oswaldo Cruz Foundation (Recife, Brazil). Mice were submitted to a deficient diet (RBD) planned to simulate the one usually ingested by low income people in north-eastern Brazil (Coutinho et al. 1997b), with 8% protein and 3.15 kcal/g. Control mice (the well-nourished groups) were fed a balanced, commercial mouse chow (Nuvital Nutrients, Colombo, Parana, Brazil), containing 22% protein and 3.69 kcal/g. Diets were given in pellet form.

Experimental infection

Mice were submitted to percutaneous infection with 30 recently shed *S. mansoni* cercariae obtained from laboratory-raised *Biomphalaria glabrata* snails (Belo Horizonte strain). The animals were distributed into the following groups: group 1, undernourished (U); group 2, infected, undernourished (UI); group 3, well-nourished (W); group 4, infected, well-nourished (WI).

Animals were fed their respective diet from 30 days before infection to the end of the experiment. Infection was confirmed in each mouse by the detection of *S. mansoni* eggs in the feces 50 days after cercarial exposure.

SEA preparation

To obtain *S. mansoni* eggs, Swiss Webster mice were infected with 100 *S. mansoni* cercariae of the Feira de Santana strain. Mice were killed 7–8 weeks after infection, and the livers were homogenized in 1.7% saline solution. The homogenate was then filtered and centrifuged at 1,300 rpm at 4°C. After purification in a percoll gradient, the eggs were homogenized using a Potter homogenizer and submitted to four cycles of freezing and thawing. The suspension was then centrifuged at 1,400 rpm at 4°C. The aqueous fraction was collected, sterilized in a gamma-ray irradiator (64,000 rad) and the protein concentration determined by a fluorescent protein assay (Stein et al. 1973).

Parasitologic and histopathologic studies

The mice were killed 16 weeks after infection. The livers were removed and weighed and pieces of the livers were placed into 4% potassium hydroxide for egg counting (Cheever 1970). Samples of liver tissue were fixed in Bouin's fixative and/or Millonig formol for histopathologic studies. After paraffin embedding, 5-μm-thick sections were stained with hematoxylin/eosin or using the picrosirius-red method for collagen (Junqueira et al. 1979).

Morphometric studies

Randomly sampled 5 μm-thick liver sections stained with picrosirius red were examined by semiautomatic morphometry using the Q500MC Image Processing and Analysis System (Leica, Cambridge, England). A total sectional area 1.34×10^{6} μm^{2} per animal was evaluated by examining all periovular granulomas present in the section. The following granuloma parameters were calculated: size, volume, volume density and numerical density. The granuloma volume density was calculated as the quotient of the total granuloma profile area to the total sectional area studied per animal. The number of granulomas per unit volume of liver was assessed by applying Weibel's formula (Weibel 1969). The sectional area of the fibrous tissue, red stained, was directly measured and calculated as a percentage of the total area examined.

Collagen assessment

The method of Bergman and Loxley (1963) was used to determine collagen concentration by the measurement of hydroxyproline content. Briefly, after fixation in neutral 10% formalin a liver fragment of 100–250 mg were hydrolyzed for 18 h in hydrochloric acid (6 N) at 110°C, neutralized in NaOH (10 N) and HCl (3 N). Samples of 100 μl were treated in a mixture of chloramine T and Ehrlich's reagent and read at 558 nm in a Hitachi 200 spectrophotometer (Hitachi, Tokyo, Japan).

Spleen cells culture

Single cell suspensions were prepared from spleens aseptically removed from mice of the four experimental groups. Spleen cell suspensions were prepared in RPMI medium (Life Technologies, GIBCO-BRL, Gaithersburg, Md.) supplemented with 10% FCS (Hyclone, Logan, Utah), L-glutamine (2 mM), vitamins, sodium pyruvate (1 mM), Hepes (10 mM), 5×10^{-5} M of 2-mercaptoethanol, and gentamycin (50 μg/ml; Sigma). For cytokine determinations, spleen cells were cultured in 24-well plates and stimulated with 1 μg/ml concanavalin A (ConA, Sigma) or *S. mansoni* egg antigen (SEA) (10 μg/ml). Cell-free supernatants were collected after 72 h of incubation and stored at −20°C for cytokine analysis. To evaluate the proliferative response, splenocytes were plated in 96-well plates at 4×10^{5}/well in a final volume of200 μl, and triplicate wells were stimulated with ConA or SEA for 72 h, as described in the figure legends. After pulsing with 1 μCi of [methyl-^{3}H] thymidine (Amersham, Little Chalfont, England) for 12 h, proliferation was assessed by the measurement of ^{3}H thymidine uptake in a β-plate counter (Packard, Meriden, Conn.).

Cytokine and antibody determinations

Levels of IFN-γ, IL-5 and IL-4 in culture supernatants were determined by sandwich ELISA, using antibody pairs and recombinant cytokines from PharMingen, following the manufacturer's instructions, followed by streptavidin-peroxidase (Sigma). Reaction was developed using the 3,3′, 5,5′-tetramethylbenzidine (TMB) peroxidase substrate (Kinkergaard and Perry Laboratories, Gaithersburg, Md.) and read at 450 nm. Anti-SEA isotype production was evaluated by ELISA using microtiter plates (Nunc MaxiSorp plates) coated overnight at 4°C with 3 μg/ml SEA in 0.02 M sodium carbonate pH 9.6. After incubation for 2 h at room temperature with sera from different experimental groups, biotinylated isotype-specific anti-mouse IgG1, IgG2a, IgG2b or IgG3 antibodies (PharMingem) were added for 45 min, followed by streptavidin-peroxidase conjugate for 30 min (Sigma). Reaction was developed using TMB substrate, as described above.

Statistical analyses

Data were analyzed using Student's *t*-test or the Kruskal-Wallis non-parametric test, as indicated in the text. Differences were considered significant when $P < 0.05$. The analyses had been carried through with the aid of Software Prism 3.0, (Graph Pad Software, San Diego, Calif.).

Results

Infection of undernourished and well-nourished mice

The average weekly protein intake of infected undernourished mice was significantly smaller than that of infected well-nourished mice (mean ± SE UI: 1.49 ± 0.10;

Table 1 Morphometric study in livers of undernourished and well-nourished *Schistosoma mansoni*-infected mice. *UI* Undernourished infected mice, *WI* well-nourished infected mice. Numbers represent means ± SD of nine mice per group

	UI	WI
Granuloma		
Volume (μm^3)	878,686.4 ± 287,800.7	1,148,014.0 ± 473,258.4
Volume density	0.187 ± 0.035	0.204 ± 0.033
Numerical density (1/mm^{-4})	39.28 ± 14.80	28.36 ± 4.65
Fibrosis (%)	20.47 ± 7.13	26.88 ± 9.27

WI: 6.93 ± 0.44, $P < 0.001$). The caloric intake of mice submitted to the hypoproteinic diet was also smaller than of well-nourished mice (UI: 58.55 ± 4.04; WI: 116.18 ± 7.43, $P < 0.0001$). Well-nourished infected mice had egg numbers/g of liver similar to those of undernourished infected mice (UI: 4.01; WI: 6.36 geometric means. $P > 0.05$).

Egg granulomas and liver fibrosis development

Liver sections of undernourished mice had smaller egg granulomas than those observed in liver sections of well-nourished animals 4 months after *S. mansoni* infection (Table 1, Fig. 1). When morphometric measurements were made for granuloma volume, the values for the well-nourished infected group were greater than those for the undernourished infected group (Table 1, $P = 0.04$). However, when numerical density (Nn) was considered, the values for the undernourished infected group were greater than those for the well-nourished infected group ($P = 0.02$). In terms of volume density (Vv), which means the total volume occupied by the granulomas within a given unit of volume, the values for the well-nourished infected group were greater than for the undernourished infected group, but the difference was not significant ($P = 0.061$). *S. mansoni* infection caused an increase in the collagen content in the livers of undernourished and well-nourished mice (Fig. 2).

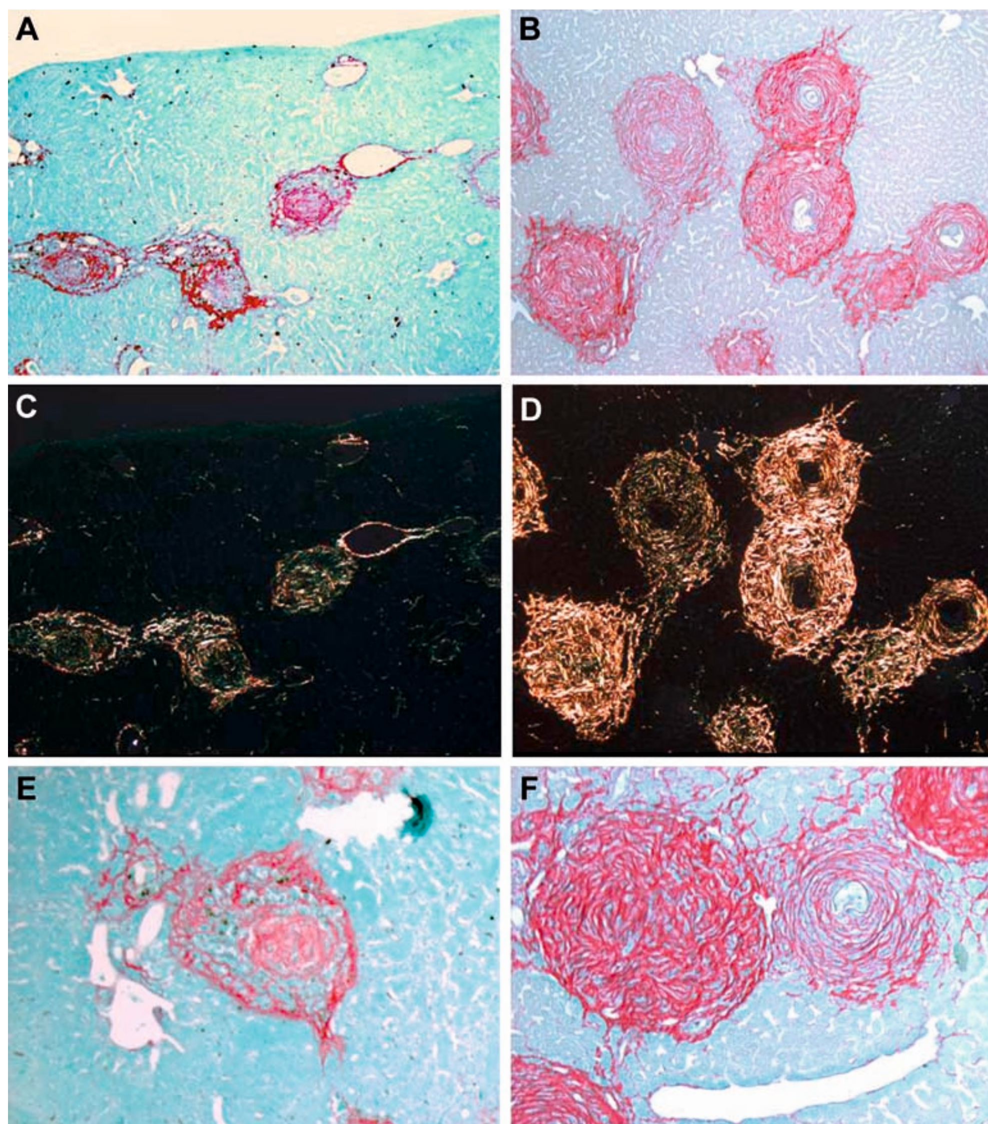

Fig. 1A–F Periovular schistosomal granulomas in liver sections of mice 4 months after *Schistosoma mansoni* infection. **A**, **C**, **E** Liver section of an undernourished mouse showing small, periovular granulomas sparsely distributed throughout the hepatic parenchyma. **B**, **D**, **F** Liver section of a well-nourished mouse showing several granulomas, with dense collagen deposition. Picrosirius red in bright field (**A**, **B**, **E, F**) and polarization (**C, D**). **A–D** 100×; **E, F** 200×

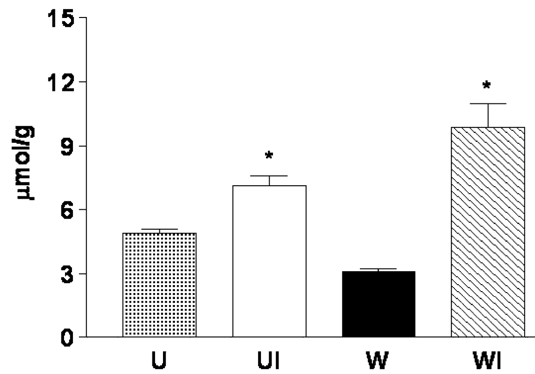

Fig. 2 Quantification of hydroxyproline in the livers of well-nourished and undernourished mice. Undernourished infected (*UI*) or uninfected (*U*) mice and well-nourished infected (*WI*) or uninfected (*W*) mice were killed 4 months after *S. mansoni* infection. The collagen content in the livers of mice from the different experimental groups was estimated by measurement of hydroxyproline. The results show the mean ± SD of seven to eight mice per group. * $P < 0.005$ when compared to uninfected groups

However, the undernourished infected mice had significantly less liver collagen than the well-nourished ones (Fig. 2, $P < 0.001$).

Lymphoproliferative response in *S. mansoni*-infected mice

In order to investigate a possible contribution of the immune responses of infected mice to the development of fibrosis in schistosomiasis mansoni, the lymphoproliferative responses of mice from different groups were compared 4 months after *S. mansoni* infection (Fig. 3). Spleen cells from infected mice of the undernourished and well-nourished groups had similar proliferative responses to ConA when compared to uninfected mice (Fig. 3a). The proliferation index of the spleen cells of undernourished infected mice stimulated in vitro with ConA was also similar to that of well-nourished infected mice (Fig. 1a). In the four experimental groups, the proliferative responses of splenocytes to SEA were low, and the differences observed were not significant (Fig. 3b).

Cytokine production by spleen cells after *S. mansoni* infection

The production of Th1 (IFN-γ) and Th2 (IL-4 and IL-5) cytokines was evaluated 4 months after *S. mansoni* infection. Spleen cells from both undernourished and well-nourished infected mice produced low levels of IFN-γ on stimulation with ConA and undetectable levels of this cytokine on stimulation with SEA (Table 2). The IL-5 levels produced by the spleen cells from undernourished and well-nourished infected mice after ConA and SEA stimulation were also similar (Table 2). IL-4 production was not detected in cell cultures from mice in any experimental group.

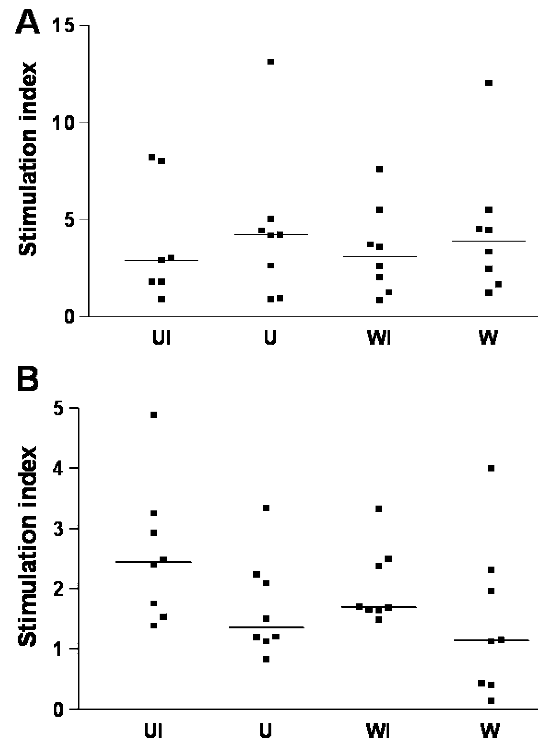

Fig. 3A, B Comparison of the lymphoproliferative response of spleen cells from *S. mansoni* infected mice. Spleen cells from undernourished infected (*UI*) or uninfected (*U*) mice and well-nourished infected (*WI*) or uninfected (*W*) mice were obtained 4 months after *S. mansoni* infection. Splenocytes were stimulated in vitro with ConA (**A**) or SEA (**B**). Lymphoproliferation was assessed by the measurement [^{3}H] TdR uptake after 72 h of cell culture. The stimulation indices of spleen cell cultures from individual mice (eight per group) were calculated by the ratio between stimulated and non-stimulated wells

SEA-specific antibodies

At 4 months after *S. mansoni* infection, the isotype profile of undernourished and well-nourished infected mice was investigated. The levels of SEA-specific IgG1, IgG2a, IgG2b and IgG3 antibodies detected in the serum of undernourished infected mice were two- to four-fold lower than those of the well-nourished infected controls (Fig. 4; $P < 0.05$). IgG1 and IgG3 were the

Table 2 Cytokine production by spleen cells in undernourished and well-nourished *S. mansoni* infected mice. *UI* Undernourished infected mice, *WI* well-nourished infected mice. Numbers represent means ± SD of individual cultures of 7–8 mice per group (pg/ml)

	Medium		ConA		SEA	
Cytokine	UI	WI	UI	WI	UI	WI
IFN-γ	ND	22	102 ± 107	72 ± 58	ND	ND
IL-5	ND	ND	390 ± 180	304 ± 157	30 ± 25	40 ± 27

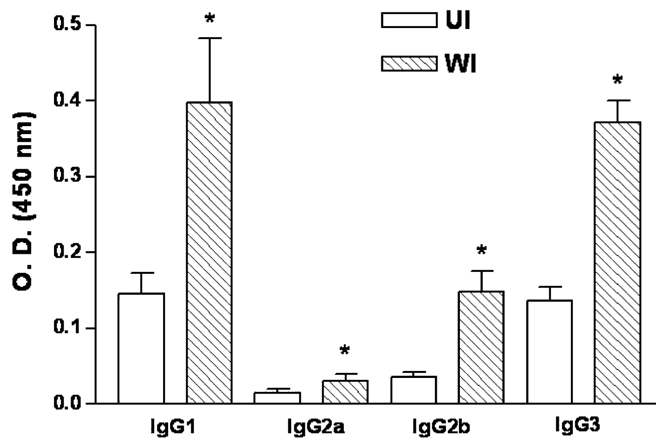

Fig. 4 Titers of SEA-specific antibodies in *S. mansoni* infected mice. Sera from infected mice fed a low-protein (*UI*) or a balanced (*WI*) diet were obtained 4 months after infection. Titers of IgG1, IgG2a, IgG2b or IgG3 anti-SEA antibodies were determined by ELISA. Data represent the mean ± SD of seven to eight mice per group. * $P < 0.005$

predominant IgG isotypes present in the sera of mice from both experimental groups (Fig. 4).

Discussion

According to previous investigators, both cellular and humoral immune responses may be affected by the host's nutritional state (Cunningham-Rundles 1982; Beisel 1987; Chandra 1999). The results presented here show that undernourished infected mice have similar proliferative and cytokine responses to well-nourished infected animals, but produce antibody levels significantly lower than those of the well-nourished infected controls. This type of immune response is associated with a less severe pathology in undernourished mice (granulomas reduced in number and size) when compared to controls.

In the present work, morphometric analyses showed similar results to those obtained previously by our group (Coutinho et al. 1997b), except for the numerical density of the granulomas, which reached only marginal differences ($P = 0.061$) between the group means. Since Nn is influenced not only by Vv, but also by the number of profiles, shape and distribution of the granulomas, it is possible that in this work the smaller tissue compartment evaluated could have influenced the results.

In a study carried out by Ghanem et al. (1987), patients with the hepatoesplenic clinical form of schistosomiasis had an increased production in the IgG synthesis and higher levels of circulating immune complexes. The demonstration of IgG deposits in the Disse's spaces in liver sections of patients with advanced Symmers' fibrosis caused by *S. mansoni* infection (Grimaud et al. 1977) suggests that a greater production of SEA-specific IgG antibodies contributes to the aggravation of the pathological process. Thus, it is possible that the higher levels of antibodies found in infected well-nourished mice contribute to a higher collagen deposition in the liver sections observed in these mice, in comparison with undernourished animals.

The low antibody responses in undernourished infected mice could be related to a lower antigenic load compared to that of well-nourished infected mice. Undernourished and well-nourished mice have similar numbers and sex ratios of worms (unpublished results). However, worms recovered from undernourished infected mice show morphological changes in their internal and external structures and exhibit a low fecundity when compared to parasites recovered from well-nourished hosts (Neves et al. 2001; Oliveira et al. 2003). In low-protein fed mice, the low fecundity detected in these parasites probably affects the production of eggs and of periovular granulomas, as well as the progression to liver periportal fibrosis (Coutinho et al. 1997b). Although it is not possible to predict the magnitude of the antigenic stimulus, the fact that the egg number per gram of tissue was similar between the two infected groups suggests that antigenic load per body mass was similar.

The proliferative response of spleen cells obtained from well-nourished and undernourished mice to SEA was low. Similarly, the levels of IFN-γ were low or undetectable upon stimulation with ConA or SEA. These findings are in agreement with data in the literature showing the suppression of IFN-γ, an antifibrosant cytokine, and the proliferative responses in chronic *S. mansoni* infection (Cheever et al. 2000; Fallon 2000). It is likely that the modulation of the immune response may induce alterations in the size, shape and composition of periovular granulomas (Andrade and Warren 1964). Concomitantly, the fibrotic reaction in the liver is accelerated by an increase in collagen deposition (Grimaud et al. 1977) and linked to a Th2 response (Yamashita and Boros 1992; Cheever et al. 1994; Chiramonte et al. 1999).

It is well known that malnutrition has several effects on mechanisms of repair, such as decreased fibroblast proliferation, collagen and albumin production and wound remodeling (Mora 1999). Recent data from our laboratories suggest that the host's nutritional status plays a role in connective tissue changes in hepatic schistosomiasis in mice (Coutinho et al. 2004). Therefore, the decreased fibrosis observed here in undernourished infected mice may result from an impaired repair mechanism, a lower antigen load or low antibody production due to low protein synthesis in a low-protein diet host.

The finding that undernourished mice develop less severe disease after *S. mansoni* infection may seem paradoxical if one takes into account that undernourished infected hosts usually present higher susceptibility to other pathogens. However, in the case of *S. mansoni* infection, the parasites do not multiply in the host and the pathology results from the immune response against egg antigens, which may be depressed in situations of low protein intake. The mechanisms by which malnutrition affects the pathology resulting from *S. mansoni* infection remain to be determined.

Acknowledgements Financial support came from CNPq and PRONEX. The authors wish to thank Andrea Ferreira de Barros and Roni Evêncio de Araújo for technical assistance and Ulisses Montarroyos who kindly helped with the statistical analysis.

References

Andrade ZA, Cheever AW (1993) Characterization of the murine model of schistosomal hepatic periportal fibrosis (''pipestem'' fibrosis). Int J Exp Pathol 74:195–202

Andrade ZA, Warren KS (1964) Mild prolonged schistosomiasis in mice: alterations in host response with time and the development of portal fibrosis. Trans R Soc Med Hyg 58:53–57

Beisel WR (1987) Role of nutrition in immune system diseases. Compr Ther 13:13–19

Bergman I, Loxley R (1963) Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. Anal Chem 35:1961–1965

Burt AD (1993) Cellular and molecular aspects of hepatic fibrosis. J Pathol170:105–114

Chandra RK (1997) Nutrition and the system: an introdution. Am J Clin Nutr 66:460–463

Chandra RK (1999) Nutrition and immunology from the clinic to cellular biology and back again. Proc Nutr Soc 58:681–683

Cheever AW (1970) Relative resistance of the eggs of human schistosomes to digestion in potassium hydroxide. Bull World Health Organ 43:601–603

Cheever AW, Williams ME, Wynn TA, Finkelman FD, Seder RA, Cox TM, Hieny S, Caspar P, Sher A (1994) Anti-interleukin-4 treatment of *Schistosoma mansoni*-infected mice inhibits development of T cells and non-B, non-T cells expressing Th2 cytokines while decreasing egg-induced hepatic fibrosis. J Immunol 153:752–759

Cheever AW, Hoffmann KF, Wynn TA (2000) Immunopathology of schistosomiasis mansoni in mice and men. Immunol Today 21:465–466

Chiramonte MG, Schopf LR, Neben TY, Cheever AW, Donaldson DD, Wynn TA (1999) IL-13 is a key regulatory cytokine for Th2 cell-mediated pulmonary granuloma formation and IgE responses induced by *Schistosoma mansoni* eggs. J Immunol 162:920–930

Coutinho EM (1980) Patobiologia da desnutrição nas doenças parasitárias. Mem Inst Oswaldo Cruz 75:63–76

Coutinho EM, Barbosa FS, Barbosa JM, Pessoa D, Pinto RF, Oliveira PA, Rodrigues BA (1972) Inquérito clínico-nutricional e antropométrico preliminar, em áreas endêmicas de esquistossomose mansonica, no nordeste do Brasil. Rev Soc Bras Med Trop 6:211–236

Coutinho EM, Abath FGC, Barbosa CS, Domingues ALC, Melo MCV, Montenegro SML, Lucena MAF, Romani SAM, Souza WV, Coutinho A (1997a) Factors involved in *Schistosoma mansoni* infection, in rural areas of northeast Brazil. Mem Inst Oswaldo Cruz 92:707–715

Coutinho EM, Souza MM, Silva LM, Cavalcanti CL, Araujo RE, Barbosa AAJr, Cheever AW, Andrade ZA (1997b) Pathogenesis of schistosomal ''pipestem'' fibrosis: a low-protein diet inhibits the development of ''pipestem'' fibrosis in mice. Int J Exp Pathol 78:337–342

Coutinho EM, Oliveira SA, Barros AF, Silva LM, Barbosa AAJr, Andrade ZA (2004) Host nutritional status as a contributory factor to the remodeling of schistosomal hepatic fibrosis. Mem Inst Oswaldo Cruz (in press)

Cunningham-Rundles S (1982) Effects of nutritional status on immunological function. Am J Clin Nutr 35:1202–1210

Czaja MJ, Weiner FR, Takahashi S, Giambrone MA, Van Der Meide PH, Schellekens H, Biempka L, Zern MA (1989) Gamma-interferon treatment inhibits collagen deposition in murine schistosomiasis. Hepatology 10:795–800

Fallon PG (2000) Immunopatology of schistosomiasis: a cautionary tale of mice and men. Immunol Today 21:29–34

Ghanem AM, Boctor FN, Bassily S, Shaheen H, Garges L (1987) Circulating immune complex levels in patients with schistosomiasis and complication. Trans R Soc Trop Med Hyg 81:773–777

Grimaud JA, Borojevic R, Bradrawy NE (1977) IgG deposits and Disse's space pathology in human schistosomal liver. Experientia 33:1078–1079

Junqueira LCU, Bignolas G, Brentani R (1979) Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochemistry 1:447–455

Machado IB (1983) Imunocompetencia en malnutrition. Rev Soc Venezol Gastroenterol 37:157–166

Mora RJF (1999) Malnutrition: organic and functional consequences. World J Surg 23:530–535

Neves RH, Machado-Silva JR, Pelajo-Machado M, Oliveira SA, Coutinho EM, Lenzi HL, Gomes DC (2001) Morphological aspects of *Schistosoma mansoni* adult worms isolated from nourished and undernourished mice: a comparative analysis by confocal laser scanning microscopy. Mem Inst Oswaldo Cruz 96:1013–1016

Oliveira SA, Barbosa-JR AA, Gomes DC, Machado-Silva JR, Barros AF, Neves RH, Coutinho EM (2003) Morphometric study of S *chistosoma mansoni* adult worms recovered from undernourished infected mice. Mem Inst Oswaldo Cruz 98:623–627

Stein S, Bohlen P, Stone J, Dairman W, Udenfried S (1973) Amino acid analysis with fluorescamine at the picomole level. Arch Biochem Biophys 155:155–202

Warren KS (1966) The pathogenesis of clay-pipe stem cirrhosis in mice with chronic Schistosomiasis mansoni, with a note on the longevity of the schistosomes. Am J Pathol 49:477–489

Weibel ER (1969) Stereological principles for morphometry in electron microscopic cytology. Int Rev Cytol 26:235–302

Wynn TA, Cheever AW (1995) Cytokine regulation of granuloma formation in schistosomiasis. Curr Opin Immunol 7:505–511

Yamashita T, Boros DL (1992) IL-4 influences IL-2 production and granulomatous inflammation in murine schistosomiasis mansoni. J Immunol 149:3659–3664